Formulation, Optimization, in Vivo Pharmacokinetic, Behavioral and Biochemical Estimations of Minocycline Loaded Chitosan Nanoparticles for Enhanced Brain Uptake

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The minocycline hydrochloride (MH), at higher doses, is useful in the treatment of neurodegenerative disorders and owing to its antioxidant potential, it may have nootropic effects. MH loaded nanoparticles (MHNP) were coated with tween 80 (cMHNP) to improve its brain uptake followed by their optimization employing two factor-three level (2³) central composite design (CCD) in order to minimize particle size and maximize drug entrapment efficiency (DEE) and validated. The optimized formulations were further subjected to in vitro drug release study; in vivo biodistribution studies in male wistar rats. The pharmacodynamic study was carried out using elevated plus maze (EPM) and Morris water maze (MWM) behavioral models for nootropic activity in swiss albino mice; and biochemical estimations (acetylcholine esterase, reduced glutathione, malondialdehyde and brain nitrite level). After intravenous (i.v.) administration, the concentration of MH in brain of cMHNP (6.21±0.64 µg/mL) treated rats was significantly higher with MH solution treated (0.70±0.06 µg/mL) as well as MHNP (1.03±0.12 µg/mL) treated animals. Pharmacodynamic studies revealed a significant improvement in memory of MH, MHNP and cMHNP treated swiss albino mice than saline treated control group. However, cMHNP revealed maximum decrease in transfer latency (TL) in EPM and maximum increase in time spent in target quadrant (TSTQ) in MWM. Although cMHNP did not produce significant change in brain acetylcholinesterase, but, significantly increased reduced glutathione, malondialdehyde and reduced brain nitrite level as compared to saline, MH solution and MHNP treated groups. The results suggest that cMHNP is a promising candidate for improved brain uptake of MH with better nootropic effect.

Key words minocycline; optimization; elevated plus maze; Morris water maze; malondialdehyde; glutathione

Minocycline hydrochloride (MH), a semi-synthetic tetracycline, is reported to be only tetracyclines effective in various neurodegenerative diseases like multiple sclerosis, Huntington’s disease, Alzheimer’s disease etc. and is also effective as an antioxidant, neuroprotective, antiviral and anti-inflammatory agent.1,2 MH acts by inhibiting the release of cytochrome c from the mitochondria, inhibition of caspase 1, 3 and inducible nitric oxide transcriptional up-regulation and activation, reactive microgliosis, activation of p38 mitogen-activated protein kinase (MAPK) and down-regulation of pro-inflammatory cytokines.3,4 Although among the tetracyclines, the levels of MH in the brain were almost three folds to that of doxycycline,5 it poorly crosses the blood–brain barrier (BBB) due to its low lipophilicity and half-life.3,6 MH is routinely administered to humans for the treatment of infectious and inflammatory diseases, the doses are much lower (3 mg/kg) than those reported to elicit neuroprotectant activity in experimental models (22–100 mg/kg multiple times daily either orally or intraperitoneally)5,6. Central nervous system (CNS) drug delivery has always been a challenging task for the researchers. The nanoparticulate drug delivery system has been used for targeting the drugs to brain, as solid lipid nanoparticles or coating them with non ionic surfactants like tween 80, poloxamine 908 etc.57–100 In order to improve the brain uptake of MH, nanoparticulate drug delivery system may serve as an efficient tool (with suitable bio-fabrication) to maximize BBB permeability.

Chitosan, one of the most abundant biopolymer, poly[β-(1,4)-2-amino-2-deoxy-d-glucopyranose], possesses unique structural feature. It has primary amine group of the glucosamine residues at the C-2 position, which confers important functional properties to it that can be suitably utilized for biofabrication into nanoparticles.11 Moreover, chitosan is a linear polyamine containing a number of free amine groups that are readily available for cross linking. Its cationic nature allows ionic cross linking with multivalent anions, whereas its mucoadhesive character increases residual time at the absorption site.12 Chitosan is a promising candidate for preparation of nano and micro-particulate drug delivery systems owing to its better stability, low toxicity, simple and reproducible preparation methods and providing versatile routes of administration as drug delivery carrier.8,13 It also avoids the use of hazardous organic solvents during fabrication, due to its in aqueous acidic solution. That is why, chitosan was selected as a polymeric carrier for nanoparticles formulation in the present study.

In present investigation, it was aimed to formulate and optimize MH loaded nanoparticles and evaluate them for enhanced brain uptake by biodistribution studies and to study the expected nootropic potential of MH by evaluating pharmacodynamic studies and biochemical estimation. The work has a novel approach of use of an antibiotic for its antioxidant and nootropic potential.

Experimental

Materials MH was received as a gift sample from Ranbaxy Laboratories Ltd., Gurgaon (Haryana), India. Chitosan (degree of deacetylation 84.97%, molecular weight 122978 viscosity molecular weight units) was obtained as a gift sample from Central Institute of Fisheries Technology, Kochi, India. Other chemicals used were of suitable analytical grade.

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Young male wistar rats (230–250 g, 8 week old) and young swiss albino mice (25–30 g, 6 week old) were procured from Disease Free Small Animal House, LLRVAS, Hisar (Haryana) India. Animals were acclimatized for seven days to laboratory conditions before experimentation. The animals were kept in groups of three, in plastic cages with soft bedding, under standard conditions of light and dark cycle, with free access to food and water. All the experiments were carried out in daytime between 08:00 a.m. and 04:00 p.m. The experimental protocols were approved by Institutional Animals Ethics Committee (IAEC) and the animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Registration No. 0436). All the possible efforts were made throughout the study to minimize the animal discomfort. The minimum number of animals (n=6) were used in the study for statistical significance.

Preparation of Chitosan Nanoparticles The chitosan nanoparticles were prepared by ionotropic gelation method with minor modifications. Limits and ranges for chitosan and tween 80 were set for the optimization studies using a two factor three level central composite design (CCD) as summarized in Table 1. The chitosan and tri-poly phosphate pentasodium (TPP) were combined electro-statically entrapping the drug followed by coating with tween 80. Briefly, to specified concentration of chitosan solution (CS) in 2% acetic acid solution (pH 5.6) containing constant amount of drug (50 mg), an aqueous solution of TPP (Chitosan:TPP=3:1) was added dropwise and stirred for 1 h using magnetic stirrer followed by addition of specified concentration of tween 80 with continuous stirring. Thereafter, the dispersion was centrifuged at 15000 rpm for 60 min at 10°C so as to collect nanoparticles as pellets. To check the reproducibility of the results, all the pellets were washed, re-dispersed in 20 mL of HPLC grade water and sonicated using probe sonicator for 2 min. Two milliliters of this suspension was further diluted 10 times, sonicated and used for the determination of particle size and zeta potential. The undiluted nanoparticles were lyophilized using lyophilizer (Alpha 2-4 LD Plus CHRIST, Germany) after adding 10% mannitol as cryoprotectant. The cryoprotectant was required to prevent particle aggregation during lyophilization, which upon reconstitution offers improved re-dispersibility, injectability and syringeability. Various batches were formulated employing varying concentrations of chitosan and tween 80 (Table 1).

Experimental Design A CCD (with α=1) using three levels each of the two factors factor X1 (chitosan concentration) and X2 (tween 80 concentration), were used for further investigations as required by the design, and the factor levels were suitably coded. Table 1 summarizes the 13 experimental runs studied employing different levels of the two factors i.e., chitosan concentration and tween 80 concentration. Each experiment was conducted in triplicate and the values were reported as mean±S.D.

Physicochemical Characterization of Nanoparticles. Particle Size, Zeta Potential and Size Distribution The average hydrodynamic diameter, polydispersity index (PDI) and zeta potential of the formulated nanoparticles were determined by dynamic light scattering (DLS) analysis using Zeta Sizer Nano ZS90 (Malvern Instruments Limited, U.K.) equipped with a 4.0 mW He–Ne laser operating at 633 nm. The samples of nanoparticle dispersion were placed in disposable cuvettes for size and zeta potential measurements. All measurements were carried out after dispersing the nanoparticles in appropriate volume of HPLC grade water at 25°C, at detection angle of 90° (for size and PDI) and 120° (for zeta potential).

Drug Entrapment Efficiency The supernatant liquid obtained after centrifugation (pellet formation) was collected; filtered through 0.45 μm syringe filter, and the amount of drug present was analyzed at λmax=345 nm using UV spectrophotometer (Cary 5000, Instrument version No. 1.12). The amount of drug in supernatant (w) was then subtracted from the total amount of drug added (W, 100 mg in this case). The percentage drug entrapment efficiency (DEE) was calculated using the formula:

\[ \text{DEE} = \frac{\text{total amount of MH (W)}}{\text{total amount of MH (W)}} \times 100 \]

Optimization Data Analysis Design Expert Software ver. 8.0.7.1 (Stat-Ease, Minneapolis, MN, U.S.A.) was employed to fit full second order polynomial equations with added interaction terms to correlate dependent variables (responses) with the independent variables (factors). The response variables considered for systematic optimization were particle size and DEE. The optimum formulations’ prognosis was conducted by locating the feasible space followed by an exhaustive grid search to obtain the possible solutions. The optimum solution of tween 80 coated minocycline hydrochloride nanoparticles (cMHNP) was located by Design Expert software using the overlay plot and formulated. The corresponding formulation without tween 80 coating (MHNP) was also formulated to compare it with tween 80 coated formulations.

Electron Microscopic Examination The optimized batch was formulated and examined under a transmission emission microscope (Morgagni 268 D, Fei Co., the Netherlands, operated at 60 kV) to study the morphology of NPs. The Karnovsky’s fixed samples were washed in phosphate buffered saline, fixed with 2.5% glutaraldehyde, post fixed with 1% osmium tetroxide and embedded in Epon. Thin sections of the tissues were cut, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Morgagni 268 D, Fei Co., the Netherlands, operated at 60 kV) to study the morphology of NPs.

Table 1. Composition of Various Chitosan-TPP Nanoparticle Formulations Prepared as per Experimental Design

<table>
<thead>
<tr>
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<th>Trial No.</th>
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Translation of coded levels in actual units

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<th>Coded level</th>
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<th>X2: Tween 80 (%)</th>
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buffer (0.1 M, pH 7.4, 6°C) and post fixed for 2 h in 1% osmium tetroxide in the same buffer at 4°C. The specimens were sonicated to prevent aggregation and dehydrated with graded acetone. The NPs were stained with uranyl acetate and lead acetate and examined under transmission electron microscope.

**Diffusional Scanning Calorimetry (DSC)** The thermogram of pure chitosan, pure MH, mannitol and optimized freeze dried cMHNP was carried out using DSC. An empty aluminum pan was used as a reference. The sample (5 mg) was placed onto standard aluminum pan, crimped and heated at the speed of 10°C/min from 40 to 450°C with continuous purging of nitrogen (20 mL/min). The empty sealed pan was used as reference.

**FT-IR Spectroscopy** In order to evaluate any chemical interaction between chitosan and MH, spectra of the pure chitosan, pure MH and optimized cMHNP were obtained (by KBr Pellet Method) on FT-IR (Spectrum RX, FTIR system, PerkinElmer, U.S.A.).

**In Vitro Release Study of Formulated Nanoparticles** In vitro drug release study from the optimized cMHNP and MHNP was carried out using the equilibrium dialysis technique at 37±1°C. Nanoparticles (equivalent to 1 mg MH) were suspended in 5 mL PBS (pH 7.4) and placed in a dialysis membrane bag (Himedia, MWCO, molecular mass cut off 12000–14000, pore size 2.4 nm). The membrane bag containing nanoparticle suspension was placed in 300 mL PBS. The agitation speed was set at 50 in order to avoid the excessive torque produced due to higher speed which may lead to rupture of the dialysis membrane. At regular intervals, 5 mL of the aliquots were collected and replaced with equal volume of fresh PBS to maintain the sink condition. The samples were centrifuged and the supernatant was analyzed to calculate the concentration of MH using UV Visible Spectrophotometer (SPECTROD 200-Analytik Jena, 07745Jena).

**In Vivo Biodistribution Study** The brain targeting efficiency of MHNP and cMHNP (dose=100 mg/kg, intravenously (i.v.)) was evaluated by comparing the concentrations of MH in plasma, brain, liver, kidney, heart, spleen, and lungs of all groups (n=6, male wistar rats).

**Study Design** Animals (Male Wistar rats) were randomly divided into sixteen groups consisting of six animals in each group.

- **Group 1:** Control group, physiological saline (i.v.) was administered.
- **Groups 2–6:** Pure drug MH (100 mg/kg, i.v.) was administered.
- **Groups 7–11:** MHNP (equivalent to 100 mg/kg, i.v.) was administered.
- **Groups 12–16:** cMHNP (equivalent to 100 mg/kg, i.v.) was administered.

**Experiment** The animals of pure drug (MH) and test groups (MHNP and cMHNP) were sacrificed by decapitation at five different time intervals (1, 2, 8, 16, 24 h, respectively) after administration of initial dose at zero hr. Blood samples were collected in heparinized tubes and centrifuged to collect the plasma and stored at −20°C. The brain, liver, lungs, spleen, kidneys, and heart were quickly removed and weighed. The removed tissues/organs were properly washed (at least thrice) with phosphate buffer to remove excess surface blood so as to eliminate false positive results and were stored at −20°C until further processing. Before processing, the samples were removed from the deep freezer at the time of analysis, kept at the room temperature for at least 30 min before analysis.

For plasma samples, 20 µL tri-fluoroacetic acid (TFA) was added to 100 µL plasma and after mixing for 30 s, centrifuged at 5400×g for 5 min. The supernatant thus obtained was collected and analyzed for the amount of drug present using HPLC (Waters 600 Plus, Waters Corporation, Milford, Massachusetts, U.S.A.). For all organs (brain, liver, kidney, heart, spleen and lungs), the weighed tissue samples were rinsed, whirled and homogenized with 20 volumes of dimethyl sulfoxide, followed by 4 h extraction at room temperature protected from light. The mixtures were then centrifuged at 16000×g for 5 min. The concentration of MH in the supernatant (containing the extracted MH) was measured by HPLC. The chromatographic conditions were: stationary phase, RP8 column (250×3 mm, i.d.) 150×3.9 10 µm Waters RP phenyl; mobile phase, acetonitrile–HPLC grade water–perchloric acid (26:74:0.25) whose pH was adjusted to 2.5 with 5 M NaOH and the flow rate of 1 mL/min. The injection volume was 20 µL and the UV detector was set at 350 nm. The mobile phase was filtered through a 0.22-µm membrane filter and degassed using an ultrasonicator. The experiments were carried out at room temperature of about 20°C. The extraction efficiency or recovery rate was also tested. For this, control tissues (MH free) were collected and homogenized. The homogenate was spiked with a known quantity of MH and mixed. After 30 min, the sample was extracted and analyzed with the same procedure described as above.

**Pharmacodynamic Study, Study Design** The experimental animals (male swiss albino mice) were randomly divided in the following groups having 6 mice in each group:

- **Groups for elevated plus maze (EPM)**
  - **Group 1:** Control Group; normal saline was administered once daily for 7 consecutive days.
  - **Group 2:** Positive Control Group; Piracetam (400 mg/kg, intraperitoneally (i.p.)) was administered once daily for 7 consecutive days.
  - **Group 3:** Pure drug (MH) solution (100 mg/kg, i.v.) was administered once daily for 7 consecutive days.
  - **Group 4:** MHNP (equivalent to 100 mg/kg MH, i.v.) was administered once daily for 7 consecutive days.
  - **Group 5:** cMHNP (equivalent to 100 mg/kg MH, i.v.) was administered once daily for 7 consecutive days.
  - **Group 6:** Control Group; normal saline was administered once daily for 7 consecutive days.
  - **Group 7 to 10:** These were similar to groups 2 to 5, except the time spent in target quadrant (TSTQ) was measured in Morris Water Maze.

**EPM Model** The EPM test is recommended as a simple method for the evaluation of learning and memory processes by measuring transfer latency (TL). The procedure, technique and end point for testing learning and memory were followed as described by the parameters. The EPM for mice consisted of two open arms (16×5 cm) and two covered arms (16×5×15 cm) extended from a central platform (5×5 cm). The maze was elevated to a height of 25 cm from the floor. On Day 1, each mouse was placed at the end of an open arm, facing away from the central platform. TL is the time taken by the animal to move from the open arm into one of the covered
arms with all its four legs. TL was recorded on the Day 6 of dosing (acquisition) for each animal. In case, the animal did not enter into one of the covered arm within 90 s, it was gently pushed into one of the two covered arm and TL was assigned as 90 s. The mouse was allowable to explore the maze for another 2 min and then returned to its home cage. Retention of this task (memory) was examined 24 h after acquisition, i.e., on Day 7 of dosing.

**MWM Model**  The MWM is used to investigate spatial learning and memory in rodents. In this model, rat’s sensory information about its surroundings especially vision and proprioception is evaluated to quantify the spatial reference memory. The method, technique and the end point for testing memory were followed with slight modifications in the parameters as described by Richard. Concisely, MWM for mice consisted of a circular pool (60 cm in diameter, 25 cm in height) filled to a depth of 20 cm with water maintained at temperature of 25°C. The water was made opaque with non-toxic white colored dye. The water tank was divided into four equal quadrants with the help of two threads on the rim of the pool, fixed at right angle to each other. An underwater platform (with top surface 6×6 cm and painted in white) was placed inside the target quadrants (Q4 in present study) of this pool 1 cm below surface of water. The position of platform was kept unaffected throughout the training session. Each animal was subjected to four consecutive trials each day with a break of 5 min for four consecutive days, during which they were allowed to escape on to the hidden underwater platform and to remain there for 20 s. During training session, the mouse was placed gently in the water between quadrants, facing the wall of pool with drop location changing for each trial, and allowed 120 s to locate underwater platform. If the mouse was unsuccessful to find the platform within 120 s, it was lead gently on to the platform and allowed to remain there for 20 s. Each animal was subjected to training trials for four consecutive days (Day 3 to Day 6), the starting position was changed with each exposure as mentioned below and target quadrant (Q4 in the present study) remained constant throughout the training session.

- **Day 1**: Q1 Q2 Q3 Q4
- **Day 2**: Q2 Q3 Q4 Q1
- **Day 3**: Q3 Q4 Q1 Q2
- **Day 4**: Q4 Q1 Q2 Q3

On the fifth day (i.e. Day 7 of drug administration), the platform was removed and animal was placed in any of the three quadrants and allowed to explore the target quadrant for 300 s. The mean time spent in the target quadrant in search of the missing platform was noted as retrieval index or memory. The observers should always stay at the same position. Due care was taken not to change the relative location of water maze with respect to other objects in the laboratory.

**Biochemical Estimations, Study Design**  The young Swiss albino mice used for behavioral study were sacrificed same day for biochemical estimation study.

**Brain Homogenate Preparation**  The mice were sacrificed by decapitation and the brain was removed. The brain samples were rinsed with saline (0.9% sodium chloride) and homogenized in phosphate buffer (pH 7.4). The homogenates were then centrifuged at 800×g for 5 min at 4°C; the supernatant centrifuged at 9000×g for 20 min at 4°C to get the postmitochondrial supernatant (also called S9 fraction; It is the supernatant fraction obtained from an organ homogenate by centrifuging at 9000×g for 20 min in a suitable medium and contains cytosol and microsomes). This supernatant was used to evaluate lipid peroxidation, reduced glutathione, catalase, acetylcholinesterase and nitrite level.

**Estimation of Lipid Peroxidation**  Lipid peroxidation is simply free radical mediated oxidative degradation of lipids. Free radicals remove electron from lipids and damage the cell. Malondialdehyde (MDA) amount is a measure of lipid peroxidation and is examined in the form of thiobarbituric acid-reactive substances (TBARS).

In this method, postmitochondrial supernatant obtained from brain homogenates (0.5 mL) is mixed with Tris–HCl (0.5 mL) and incubated at 37°C for 2 h. After the incubation, trichloroacetic acid (1 mL of 10%) was added and centrifuged at 1000×g for 10 min. To 1 mL of supernatant so obtained, 1 mL of 0.67% thiobarbituric acid was added, and the samples were kept in boiling water for 10 min. After cooling, 1 mL of HPLC grade water was added, and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were determined quantitatively using an extinction coefficient of 1.56×10^5 m^-1 cm^-1 at 532 nm and expressed as nanomole of malondialdehyde per milligram protein. Total protein concentration was estimated in brain homogenate by using a total protein kit, using semi-autoanalyser (Model C-500, Logitech), and the brain malondialdehyde content was expressed as nanomole of MDA per milligram of protein.

**Estimation of Reduced Glutathione (GSH)**  The content of reduced form of glutathione is the measure of antioxidant content of a sample. In this method, 1.0 mL of S9 fraction and 9 mL of double distilled water were mixed with 1.0 mL of 4% sulfosalicylic acid were mixed with. The samples were kept at 4°C for at least 1 h and then centrifuged at 1200×g for 15 min at 4°C. To 0.1 mL supernatant, 2.7 mL phosphate buffer (0.1 M, pH 7.4), and 0.2 mL 5,5-dithiobis-(2-nitro benzoic acid) (also called Ellman’s reagent, 0.1 mM, pH 8.0) were added to make the total volume of 3.0 mL. A yellow color was developed which was analyzed at 412 nm. GSH levels were calculated using molar extinction coefficient of 1.36×10^4 m^-1 cm^-1, and expressed as micromole per milligram protein similar to MDA content.

**Estimation of Catalase Activity**  Catalase is a primary antioxidant enzyme that prevents the oxidative damage caused by intracellular reactive oxygen species (ROS). The assay mixture contains 1.95 mL phosphate buffer (0.05 M, pH 7.0), 1.0 mL hydrogen peroxide (0.019 M), and 0.05 mL S9 fraction (10%) in a final volume of 3.0 mL and analyzed at 240 nm. Absorbance changes were noted at 240 nm. Catalase activity was then computed using the millimolar extinction coefficient of H2O2 (0.07 mm) and expressed as micromoles of H2O2 decomposed per minute per milligram protein.

**Estimation of Acetylcholinesterase**  For estimation of brain acetylcholinesterase, 0.4 mL of brain homogenate was added to a test tube containing 2.6 mL of phosphate buffer. Dithiobisnitrobenzic acid (DTNB) reagent (0.1 mL) was then added to the above mixture and absorbance was recorded at 412 nm. Acetylcholine iodide (0.02 mL) solution was added and again absorbance was noted after 15 min at 412 nm.

The rate of hydrolysis of substrate was calculated using the following formula:
Estimations of Nitrite Level To measure the nitrite level, a mixture of 1% w/v sulphamethoxazole in 5% aqueous solution of m-phosphoric acid (1 part) and 0.1% w/v N-(1-naphthyl) ethylene diamine dihydrochloride (1 part) was prepared and kept at 0°C for 60 min. Half milliliter plasma was mixed with 0.5 mL of the above mixture and kept in dark for 10 min at room temperature. The absorbance was read at 546 nm.26)

Statistical Analysis

All in vitro physico-chemical characterization and drug release study data were expressed as mean±S.D. (n=3), and the in vivo studies data as mean±S.E.M. (n=6). The difference between the groups were tested using analysis of variance (ANOVA) followed by Tukey’s post-hoc test for biochemical parameters at the level of \( p<0.05 \). The difference greater than \( p<0.05 \) was considered significant and \( p<0.001 \) was considered extremely significant and was calculated using GraphPad Instat 3 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Results

Preparation and Characterization of Chitosan Nanoparticles. Physicochemical Characterization Different concentrations (three levels) each of chitosan and tween 80 were used to optimize the best concentrations on the basis of particles size and drug entrapment efficiency (DEE). The observed values of particle size and DEE of the formulations prepared as per the experimental design are shown in Table 2. The particle size of various formulations of NPs ranged from 155.5 to 636 nm. The polydispersity index was found to be less than 0.4 and zeta potential was found have positive value ranging from 0 to +30 mV. The DEE varied from 78.6 to 91.7% and the DEE for all the batches was higher than 75%.

Exploration of Effect of Independent Variables on Characteristics of NPs Using Response Surface Methodology. It was observed from response surface (Fig. 1) that with 0.25% w/w chitosan and 1% w/w tween 80, the particle size was the maximum. When the chitosan concentration was lowered to 0.05% w/w, the particle size was minimum. At this level, tween 80 concentration had little effect on particle size which was more pronounced at the highest chitosan concentration where increase in tween 80 concentration resulted in decreased particle size. The following equation was generated for quadratic model using Design Expert software.

\[
\text{particle size} = -12.66 + 788.89X1 - 250.48X2 - 1487.5X2^2 + 10228.97X1^2 - 59.44X2^2
\]

DEE was higher at low chitosan concentration (0.05%) which reaches to a maximum of 91.7% (Fig. 2) on increasing amount of tween 80 from 1 to 2%. However, when both chitosan concentration and tween 80 concentrations were on higher side, DEE decreased which may be attributed to higher leaching of the drug through chitosan network as the surfactant may have increased the drug permeability. The equation showing the effect of independent variables on DEE generated through Design Expert is as follows

\[
\text{DEE} = 91.55 + 42.38X1 - 0.74X2 - 22.5X1X2 - 196.55X1^2 + 0.338X2^2
\]

Overlay plot (Fig. 3) for feasibility range search was utilized with a goal to maximize DEE (which ranged between 70–99.99%; least importance was given to DEE) and minimize particle size (ranging between 150 to 200 nm; maximum importance was given to particle size). The overlay plot suggested that using chitosan at 0.07% and tween 80 at 2.00% concentration may yield nanoparticles with particle size 150.00 nm and DEE of 90.368%.

In order to confirm the designs of experiments for optimization, the formulation containing 0.07% w/w chitosan and 2.00% tween 80 (optimized batch) was prepared and evaluated. The average values of the responses (i.e., particle size and DEE) were found to be 161.57±12.86 nm (Fig. 4A) and 89.63±2.66% respectively. The predicted values and observed values were compared using percent error of mean, which was found to be 7.71% (for particle size) and 0.81% (for DEE) indicating the success of optimization model. The polydispersity index varied from 0.243 to 0.394 with an average of 0.324±0.0762. The zeta potential of the optimized batch ranged between 32.8 to 33.5 mV with an average value of 33.23±0.37 mV (Fig. 4B).

Electron Microscopic Examination The transmission electron microscopy (TEM) image of the optimized batch (Fig. 5) revealed that the particles are spherical in shape with smooth surface and size of the particles was less than 150 nm. The particle size varied from 94.63 to 137.38 nm. The results were in concordance with the particle size obtained using dynamic light scattering.

DSC Study The thermogram of drug (MH), polymer (chitosan), the cryoprotectant (mannitol) and freeze dried optimized nanoparticles with mannitol incorporation (nano 1) and without mannitol incorporation (nano 2) are shown in Fig. 6. MH had sharp melting endotherm at 201.96°C, whereas the thermogram of chitosan showed a broad endotherm at 118.08°C. Mannitol showed three peaks at 168.21°C, 329.39°C and 373.38°C but sharp peak at 168.21°C. Thermogram of MH loaded nanoparticles showed no single but multiple endotherm (118.87°C-chitosan, 226.73, 333.86-mannitol). The peak exhibited by MH at 201.96°C was not visible in MH loaded nanoparticles, indicating that MH might have transformed.
from crystalline to amorphous form during the formulation of nanoparticles.

**FT-IR Spectroscopy** IR spectroscopy method was used to study the drug–polymer interactions. IR spectroscopy of drug MH shows all the characteristic peaks of functional groups present in the drug. The absence of chemical interaction between drug and polymer was confirmed by the characteristic peaks of functional groups in the IR spectra of drug, polymer and nanoparticles (Fig. 7). The characteristic peaks of MH were observed at 3479, 3349, 3270, 1650, 1601, 1306, 1223, 1372, 3080 and 1352 cm$^{-1}$; for chitosan 3441, 1156. The characteristic peaks of drug (MH) were also observed with IR spectra of MHNP which were at 3332, 1649, 1308, 1246, 1294 and 3074 cm$^{-1}$. Similarly, the characteristic peaks of chitosan were also observed at 1144 and 3332 cm$^{-1}$ in MHNP IR spectra.

**In Vitro Drug Release Study** The cumulative percent drug release of MH was 77.46±1.17% and 73.79±0.79 from...
MHNP and cMHNP respectively over a period of 24 h as shown in Fig. 8. The percentage drug release of MH was 57.24±0.95% from MHNP and 54.85±0.55% from cMHNP respectively after 2 h. The co-efficient of correlation ($R^2$) of zero order, first order, matrix model, Korsmeyer–Peppas model, Higuchi model and Baker–Lonsdale model from MHNP and cMHNP is shown in Table 3. Korsmeyer–Peppas model ($R^2=0.9999$ and 0.9873 for MHNP and cMHNP, respectively) was observed to be the best fit model indicating fickian diffusion as the mechanism of drug release from nanoparticles.

In Vivo Biodistribution Study  Biodistribution studies of optimized, tween 80 coated MH loaded chitosan nanoparticles (cMHNP), its corresponding batch without tween 80 coating (MHNP) and MH solution was carried out in male wistar rats. These different formulations were administered intravenously through tail veins to study the biodistribution of MH. The concentration of MH observed in heart, liver, kidney, spleen and lungs after 1 h, 2 h, 8 h, 16 h and 24 h has been summarized in Table 4 and the corresponding concentration of MH in plasma and brain is represented in Fig. 9. The formulation of nanoparticles and further coating resulted in decreased accumulation of drug in liver, spleen, kidney and heart. The concentrations of MH in plasma as well as brain were highest at all time points in cMHNP treated group as compared to MH treated as well as MHNP treated group. The recovery rate (extraction efficiency) was found to be in the range of 78–84%, the tissue MH concentration was within the calibration curve of MH.

Pharmacodynamic Study. EPM The effect of treatment of Piracetam, MH, MHNP and cMHNP on TL of mice in EPM is represented in Fig. 10. Pure MH ($p<0.05$), Piracetam ($p<0.01$) and cMHNP ($p<0.01$) each with the same dose significantly decreased TL of treated mice as compared to the control group mice, showing significant memory enhancing activity; whereas MHNP treated group failed to do so. No significant difference was observed in TL of cMHNP and Piracetam indicating their equivalent activity (decrease in TL). Minimum TL was observed with the administration of cMHNP suspension (TL of mice=6.54±1.62 s) compared to control group (TL of mice=19.09±2.09 s).

MWM The effect of treatment of Piracetam, MH, MHNP and cMHNP on TSTQ of mice in MWM has been depicted in Fig. 10. MH solution (TSTQ of mice=117.92±2.86 s; $p<0.05$), Piracetam (TSTQ of mice=145.69±2.63 s; $p<0.001$) and cMHNP (TSTQ of mice=141.69±2.63 s; $p<0.001$) revealed significant increase in TSTQ as compared to saline treated control group (TSTQ of mice=103.84±4.81 s). When compared to pure MH (TSTQ of mice=117.92±2.86 s), TSTQ of both Piracetam as well as cMHNP was significantly higher ($p<0.001$).

Biochemical Estimations. Effect on Lipid Peroxidation (Brain Malondialdehyde (MDA) Levels) Treatment with MH, Piracetam and cMHNP produced an extremely significant reduction in MDA levels in the brain of mice as compared to the respective controls ($p<0.001$). No significant difference ($p>0.05$) was observed among MH and Piracetam treated animals. Significantly reduced MDA level was observed in cMHNP (6.85±0.36 units) treated group compared to both MH (9.92±0.49 units) and MHNP (9.16±0.52 units) treated groups.

Effect on Reduced Glutathione (GSH) More the value of GSH, greater will be its antioxidant activity. GSH levels were significantly increased in brain of animal groups administered with Piracetam, MH, MHNP and cMHNP as compared to respective vehicle treated control group (Fig. 11) and the difference was extremely significant ($p<0.001$). No significant difference was also observed in GSH levels of MH group when compared with both MHNP treated and Piracetam treated group. On the other hand, the cMHNP group significantly increased GSH levels in brain of animals treated with MHNP and Piracetam treated group.

Effect on Catalase Activity Catalase enzyme is an index that indicates the antioxidant potential. Catalase levels were
significantly decreased in brains of animal groups administered with pure MH (53.67±2.82 units, p<0.01), Piracetam (57.61±2.13 units, p<0.001) and cMHNP (68.55±2.99 units, p<0.001) as compared to respective vehicle treated control group (Fig. 11). Whereas MHNP treated group (49.84±2.56 units) did not show any significant difference from the saline treated control group (39.42±1.98 units). No significant difference (p>0.05) were observed when MHNP treated group was compared with MH as well as Piracetam treated group. cMHNP treated group was found to differ significantly as compared to MH as well as Piracetam treated group (Fig. 11).

**Effect on Brain Acetylcholinesterase (AChE)**  The data obtained after determination of brain AChE activity has been shown in Fig. 11. All the treatments, i.e., Piracetam (45.43±2.91 units), MH pure drug (47.41±3.67 units), MHNP (43.98±4.51 units) and cMHNP (42.62±3.88 units) treated groups did not produce a significant change (p>0.05) in brain AChE activity as compared to saline treated control group (48.71±4.07 units).

**Effect on Brain Nitrite Levels**  The brain nitrite levels of different group of animals were estimated and the data obtained is shown in Fig. 11. Pure MH (12.00±0.98 units; p<0.01) and cMHNP (6.86±0.84 units; p<0.001) treated group indicated a significant reduction in brain nitrite level.
as compared to saline treated control group (20.01±1.20 units); whereas Piracetam (18.12±2.1 units) as well as MHNP (14.56±1.05 units) treatment failed to produce a significant change in brain nitrite level. A significant difference ($p<0.01$) was observed among reduction in brain nitrite level in cMHNP and MHNP treated animals. Among all the groups, the maximum reduction in brain nitrite level was observed after administration of cMHNP.

**Discussion**

The aim of the present investigation was to develop biocompatible and biodegradable novel delivery systems comprising ligand coated nanoparticles for targeting MH to brain. The coating of nanoparticles with tween 80 was proposed to offer benefits in terms of their targeting efficacy towards brain. The proposed mechanism of brain specificity is their preferential binding with Apo-E present in the plasma that would mimic low density lipoproteins (LDL), and thus bind with the LDL receptors present on brain micro vascular endothelial cells (BMEC).  

The particle size of nanoparticles is an important property to control the endocytosis rate across the brain capillary endothelial cells. All the formulated batches had an average diameter in the nanometer range varying from 155.5 to 636nm (Table 2). The particle size was found to increase with increasing drug: polymer ratio which could be attributed to the amount of polymer that was enough to maintain the stability of nanoparticles at equal molar ratio of drug and polymer and coalescence of droplet did not occur at this ratio. An increase in the drug proportion in solution resulted in reduction of CS/TPP interaction, which leads to increase in size of nanoparticles. Further, as the amount of Tween 80 was increased, the particle size was found to decrease, possibly due to formation of smaller droplets during the formation of nanoparticles and hence smaller size. In the present investigation, positive zeta potential values were detected among all the batches, which may be attributed to the presence of residual amino group which were unreacted during the interaction with negatively charged TPP molecules. Beside this, all the measured zeta potential values were highly positive reflecting their excellent physical stability. Higher zeta potential (either positive or negative) require higher energy for bringing two particles in contact with each other i.e., it possess high energy barrier in between the particles. However, proteins and electrolytes present in blood can alter surface charge (zeta potential) of nanoparticles. Therefore, their aggregation behavior may change.

The addition of tween 80 to the formulation was found to shift the polydispersity index towards zero yielding more uniform particle size distribution i.e., monodisperse particles. The mean particle size of cMHNP observed with zetasizer was greater than that obtained after TEM imaging. This difference between the results may be attributed to the dehydration of nanoparticles during sample preparation for TEM analysis. The zetasizer uses Photon Correlation Spectroscopy and measures the apparent particle size i.e., the hydrodynamic
Fig. 6. Overlay of Differential Scanning Thermogram of Polymer, Drug, Mannitol and Nanoparticle

Fig. 7. Overlay of FT-IR Spectra of Polymer, Drug and Nanoparticle
diameter which includes the hydrodynamic layers that form around the hydrophilic particles leading to overestimation of particle size.\textsuperscript{32} The lower value of PDI of optimized batch indicated the homogenous, unimodal with narrow particle size distribution particles. The TEM image indicated that the particles were uniform, segregated, spherical and sub-spherical in shape. Drug Entrapment Efficiency (DEE) was found to vary between 78.6 to 91.7% (Fig. 2). However, when both chitosan concentration and tween 80 concentrations were on higher side, DEE decreased which may be attributed to higher leaching of the drug through chitosan network as tween 80 may have improved the permeability. The DEE of the optimized batch cMHNP was found to be 89.63 ± 2.66% which was quite close to the predicted optimized value (standard error of mean = 0.81%).

Both the batches evidenced initial burst effect followed by the sustained drug release.\textsuperscript{35} The initial burst release may be due to easy drug diffusion and desorption of surface bound/free drug.\textsuperscript{35,36} The percent drug release was observed to be the greater in case of MHNP (77.46 ± 1.17%) than cMHNP (73.79 ± 0.79%) after 24 h (Fig. 8). The coating of nanoparticles with tween 80 slightly decreased the release of drug from the corresponding batch as compared to uncoated batch. An additional barrier layer may have been formed by tween 80 coating, although it was not much significant as compared to uncoated batch. The release rate in the second phase was assumed to be controlled by the diffusion of drug across the polymer matrix of the nanoparticles.\textsuperscript{35,36}

The in vitro drug release kinetics were characterized by fitting the data of in vitro—release studies of nanoparticles from various batches to standard release kinetics equations (zero order, first-order, Higuchi (\(M_t/M_\infty<0.6\)), Korsmeyer–Peppas model (\(M_t/M_\infty<0.6\)) and Baker–Lonsdale model,\textsuperscript{36} as represented in Table 3. The best fit model for the drug release data was selected on the basis of correlation coefficient. The results indicated that release of drug from nanoparticles follow Korsmeyer–Peppas model as indicated by higher \(R^2\) values and the drug release followed anomalous transport (i.e., diffusion coupled with polymer matrix relaxation) as indicated by value of \(n\) lying between 0.45 and 0.89 in Korsmeyer–Peppas equation.

In the studies of drug delivery to brain, there is a risk of a false positive result, such as the drug present outside the blood brain barrier may be included in the quantification of the drug that has entered the brain. To avoid this, the brain was thoroughly washed with PBS until there was no detectable MH in washings before homogenization. This resulted in a safe conservative estimate of the brain content of MH.\textsuperscript{19} Table 4 depicts the concentration of MH in heart, liver, kidney, spleen and lungs following administration of pure MH, MHNP and cMHNP. The concentration of drug in brain at all five time points are shown in Fig. 9. The effect of coating with the ligand on the concentration of drug in brain can be inferred by comparing the concentration in brain obtained with coated (cMHNP) and uncoated nanoparticles (MHNP). The concentration of drug in brain achieved with cMHNP was significantly higher as compared to MHNP which probably be accounted for the proposed mechanism of brain specificity in their preferential binding with Apo-E. The Apo-E present in the plasma binds with tween 80 coated nanoparticles and would lead to mimic low density lipoproteins (LDL), and

Table 3. Release Kinetics of Chitosan Nanoparticles of the Drug MH from Various Batches (Mean Value±S.D.), \(n=3\)

<table>
<thead>
<tr>
<th>Batch</th>
<th>Zero order</th>
<th>First order</th>
<th>Matrix</th>
<th>Korsmeyer–Peppas\textsuperscript{a}</th>
<th>Higuchi\textsuperscript{a}</th>
<th>Baker–Lonsdale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R^2) Value</td>
<td>(R^2) Value</td>
<td>(R^2) Value</td>
<td>(R^2) Value</td>
<td>(n) Value</td>
<td>(k) Value</td>
</tr>
<tr>
<td>MHNP</td>
<td>0.6954</td>
<td>0.7968</td>
<td>0.8695</td>
<td>0.9999</td>
<td>0.530</td>
<td>4.5355</td>
</tr>
<tr>
<td>cMHNP</td>
<td>0.7064</td>
<td>0.8023</td>
<td>0.8766</td>
<td>0.9873</td>
<td>0.481</td>
<td>5.3570</td>
</tr>
</tbody>
</table>

\(a)\) \(M_t/M_\infty<0.6\).
in turn would bind with the LDL receptors present on brain micro vascular endothelial cells (BMEC).30,31)

As compared to pure MH and MHNP, cMHNP exhibited longer circulation in blood.37) Coating by hydrophilic tween 80 modified the surface properties of cMHNP by providing a dynamic cloud of hydrophilic and neutral chains at particle surface, which repels plasma proteins.38) This will avoid NP recognition by reticuloendothelial system (RES) resulted in increased brain distribution.37)

It was also observed that a higher tissue concentration of MH was achieved in the liver with the nanoparticles in comparison to the free MH. A similar pattern of MH distribution was also observed in the spleen. It may be concluded that higher concentrations of MH were achieved in the highly perfused organs, such as liver, kidney and spleen which may be attributed to the combined activity of the circulating blood passing through the organs as well as improved particle uptake by endothelial system cells of these organs.39) Similar findings were reported for other active pharmaceutical ingredients (API's) nanoparticle formulations.40,41)

This accumulation of drug in the liver and spleen was reduced when nanoparticles were coated with tween 80. This
result supports the earlier findings. But in kidneys, administration of both MHNP and cMHNP resulted in reduced concentration of MH in comparison with the pure drug (MH). Thus formulation of NP may be an approach towards reduction of side effects of drugs having adverse/side effects on the urinary tract and/or kidneys. In case of heart, a similar pattern as that for kidney was observed. The MH concentration in different organs is shown in Table 4. ANOVA, followed by Tukey’s test for post-hoc analysis, showed that upon formulation of nanoparticles followed by coating with tween 80 were able to achieve a significantly higher brain content of MH. Thus, the null hypothesis was rejected.

Oxidative stress leads to oxidation of lipid and protein in striatum and cortex, and neurodegenerative disorders as it causes massive loss of striatal neurons and may contribute to learning and memory deficits. Administration of minocycline and its nanoparticles with and without ligand coating showed significant antioxidant activity, as indicated by decrease in lipid peroxidation and increased catalase and GSH in mice brain. cMHNP treatment resulted in improved antioxidant and hence improved pharmacodynamic activity because of improved uptake of MH by brain via this formulation. These results are in accordance with previous findings where PPAR-α activators produced neuroprotection by decreasing oxidative stress as well as reactive nitrogen species (RNS) via reduction in free radical generation and increasing antioxidant enzymes such as catalase and endogenous antioxidant GSH.

In the present investigation, pure MH, positive control Piracetam treated group and cMHNP administered batches revealed memory enhancing activity in mice as indicated by decrease in TL and increase in TSTQ; but MHNP treated group failed to show improved memory compared to saline treated control group. Moreover, the effect was more pronounced in case of cMHNP which may be attributed to enhanced brain uptake of MH via ligand coated nanoparticles as compared to pure drug and uncoated MHNP. The concentrations achieved by MHNP were insufficient to produce significant pharmacodynamic effects compared to saline treated control group. The enhanced uptake shown by in vivo biodistribution studies is further confirmed by in vivo pharmacodynamic effects.

There was no alteration in brain AChE activity after the treatment with all formulations indicating the involvement of non-cholinergic mechanisms in memory enhancing activity. In contrast to the results obtained for AChE activity, the brain nitrite levels were found to be significantly reduced by MH but not by Piracetam; and this reduction was maximum with cMHNP treatment which may be attributed to its enhanced permeation across BBB and hence enhanced nootropic activity. The pure drug as well as its nanoparticle formulations were able to decrease the nitrite levels and can protect against oxidative damage. Thus, the reduction in nitrite levels supports the involvement of respective NO inhibition in memory enhancing activity of MH and the formulated nanoparticles. The increased pharmacodynamic activity (memory enhancing activity) of ligand coated nanoparticles may be due to improved penetration through BBB. Coating of nanoparticles with tween 80 was proposed to offer benefits in terms of their targeting efficacy towards brain.

**Conclusion**

The present investigation proposed a novel nanoparticulate formulation of MH. The effect of two variables (chitosan concentration and tween 80 concentration) on two dependent variables (particle size and drug entrapment efficiency) was investigated and an optimized batch was formulated. The observed physicochemical parameters were found to be satisfactory when compared with software predicted values. The in vitro release was found to be 73.79±0.79% after 24h, indicated a controlled and sustained release profile of optimized batch (cMHNP). An enhanced brain uptake was clearly observed following i.v. administration of cMHNP. MH has noticeable learning and memory enhancement activity, which is otherwise used for its antibiotic activity. Inhibition of AChE could

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**Fig. 11. Effect of MH, MHNP and cMHNP, on Brain MDA, GSH and Catalase Level in Mice**

MDA: p value: <0.0001, F(4, 25) value: 50.273; GSH: p value: <0.0001, F(4, 25) value: 38.795; Catalase: p value: <0.0001, F(4, 25) value: 17.810; AChE: p value: 0.7968, F(4, 25) value: 0.4140; Brain nitrite level: p value: <0.0001, F(4, 25) value: 15.651.
Table 4. Concentration of MH in Heart, Liver, Kidney, Spleen and Lungs of Wistar Rats by Administration of MH, MHNP and cMHNP at Various Time Intervals (Value= Mean±S.E.M., n=6)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>Time</td>
<td>MH</td>
</tr>
<tr>
<td>1 h</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>2 h</td>
<td>1.90±0.19</td>
</tr>
<tr>
<td>8 h</td>
<td>3.70±0.45</td>
</tr>
<tr>
<td>16 h</td>
<td>3.20±0.41</td>
</tr>
<tr>
<td>24 h</td>
<td>1.32±0.17</td>
</tr>
</tbody>
</table>

Table 5. Stability Study Data for MHNP and cMHNP

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Temperature</th>
<th>MHNP</th>
<th>cMHNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4±1°C</td>
<td>25±5°C</td>
<td>37±1°C, RH 75%</td>
</tr>
<tr>
<td></td>
<td>Percent drug content&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Physical appearance</td>
<td>IR spectra</td>
</tr>
<tr>
<td>1 Month</td>
<td>35.45±0.67</td>
<td>NC</td>
<td>NT</td>
</tr>
<tr>
<td>2 Month</td>
<td>35.15±0.61</td>
<td>NC</td>
<td>NT</td>
</tr>
<tr>
<td>3 Month</td>
<td>34.13±0.13</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>1 Month</td>
<td>30.51±0.34</td>
<td>NC</td>
<td>NT</td>
</tr>
<tr>
<td>2 Month</td>
<td>29.56±0.25</td>
<td>NC</td>
<td>NT</td>
</tr>
<tr>
<td>3 Month</td>
<td>29.15±0.65</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NT, Not tested. NC, No change. <sup>a</sup> Mean±S.D., n=3. <sup>b</sup> Lump formation.
not be the possible mechanism for memory enhancement of MH. Overall antioxidant effects due to increased catalase and GSH, reduction in MDA and nitrite level. The memory enhancing effect of cMHNP was the highest among the three (MH, MHNP and cMHNP) and is a reflection of the better brain uptake of MH through cMHNP. Brain targeted nanoparticles may have nootropic activity of MH at comparatively lower dose and thus have better patient compliance. Minocycline HCl could be further explored for other neurodegenerative disorders. However, the clinical benefit to the risk ratio of the formulation so developed, will decide its future potential in the treatment of neurodegenerative disorders so as to support the findings of the present investigation.

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